Tumor-associated auto-antibodies as early detection markers for ovarian cancer? A prospective evaluation

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Abstract

Background: Immuno-proteomic screening has identified several tumor-associated auto-antibodies (AAb) that may have diagnostic capacity for invasive epithelial ovarian cancer, with AAb to P53 proteins and cancer-testis antigens (CTAGs) as prominent examples. However, the early detection potential of these AAbs has been insufficiently explored in prospective studies.

Experimental Design: We performed ELISA measurements of AAbs to CTAG1A, CTAG2, P53, and NUDT11 proteins, for 194 patients with ovarian cancer and 705 matched controls from the European EPIC cohort, using serum samples collected up to 36 months prior to diagnosis under usual care. CA125 was measured using electrochemo-luminiscence. Diagnostic discrimination statistics were calculated by strata of lead-time between blood collection and diagnosis.

Results: With lead times ≤6 months, ovarian cancer detection sensitivity at 0.98 specificity (SE98) varied from 0.19 [95% CI 0.08-0.40] for CTAG1A, CTAG2 and NUDT1 to 0.23 [0.10-0.44] for P53 (0.33 [0.11-0.68] for high-grade serous tumors). However, at longer lead-times the ability of these AAb markers to distinguish future ovarian cancer cases from controls declined rapidly; at lead times >1 year, SE98 estimates were close to zero (all invasive cases, range: 0.01-0.11). Compared to CA125 alone, combined logistic regression scores of AAbs and CA125 did not improve detection sensitivity at equal level of specificity.

Conclusions: All four AAb biomarkers showed diagnostic discrimination 0-6 months prior to ovarian cancer diagnosis, but performance waned rapidly with increasing lead time beyond six months. The added value of these selected AAbs as markers for ovarian cancer beyond CA125 for early detection may be limited.

Introduction

Cancer antigen 125 [CA125] is the currently best available biomarker for epithelial ovarian cancer, and the only marker tested in prospective screening trials so far. In randomized trials, however, the combination of CA125 with trans-vaginal ultrasonography (TVUS) provided either no reduction in ovarian cancer mortality (Prostate, Lung, Colorectal and Ovarian Cancer Screening Trial [PLCO], USA) **(1)**, or only a suggestive mortality reduction using the Risk of Ovarian Cancer Algorithm ("ROCA") algorithm, based on longitudinal changes in CA125 in serial measurements over time (United Kingdom Collaborative Trial on Ovarian Cancer Screening [UKCTOCS]) **(2)**. CA125 has relatively low sensitivity for ovarian cancer early detection, particularly for early stage disease **(3)** or in serum samples taken more than 6 months prior to symptomatic diagnosis **(4, 5)**, prompting searches for complementary biomarkers that can detect ovarian cancer in earlier clinical stages and at longer lead-times prior to usual symptomatic diagnosis.

A promising class of novel markers for early cancer detection is auto-antibodies [AAbs] against mutant, aberrantly post-processed or locally over-expressed proteins in tumors **(6-8)**. Through replication of antibody producing B-cells, AAbs could amplify a signal from antigens at very low concentrations, and at an early stage in tumorigenesis when the corresponding antigens may not themselves be detectable in the circulation.

To date, more than 80 AAbs have been investigated for ovarian cancer detection **(9)**. In our own work, we have successfully discovered first sets of AAbs with high tumor specificity among ovarian cancer patients **(10-12)**. In multi-stage discovery studies, using programmable protein microarrays containing 5,177 and 10,247 candidate antigens we identified sets of three and eleven AAbs, respectively, that were significantly associated with invasive ovarian cancer. Among these, antibodies against p53, the cancer/testis antigen CTAG-2 (also known as ESO2), and NUDT11 stood out as AAb markers with highest diagnostic sensitivity (up to 27.3 and

36.4%, respectively for serous tumors) at ≥97% specificity. A further AAb frequently reported to be associated with ovarian cancer **(13) (9)** and other tumors types **(14, 15)**, is CTAG1A (also known as NY-ESO-01). However, with the exception of two recent studies on AAbs against MUC1 (Ca15.3) **(16)** and p53 **(17)**, the early detection potential of tumor associated AAbs for ovarian cancer has been insufficiently evaluated in prospective cohort studies based on pre-diagnosis serum samples, and it is still unclear whether elevated AAb levels can be used to reliably detect ovarian cancer ahead of usual diagnosis.

To further examine the capacity of AAbs to provide early detection signals for ovarian cancer, as a possible complement to CA125, we performed a prospective analysis on a selected panel of four AAbs – against P53, CTAG1A, CTAG2 and NUDT11 – within the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort, using serum samples collected up to 36 months before diagnosis of 194 ovarian cancer patients and 705 matched control participants.

Materials and Methods

Case-control study within the EPIC cohort

We conducted a case-control study nested within the EPIC cohort – a population-based, multicenter prospective cohort study in 10 western European countries – extending an extension of an earlier study on CA125 and other early detection markers for ovarian cancer **(4, 5)**. The present study includes pre-diagnosis serum samples from all incident cases (N=197) of epithelial invasive ovarian (ICD code: C569), fallopian tube (C570) or peritoneal cancers (C480, C481, C482, C488) according to the International Classification of Diseases for Oncology (ICD) with available data on tumor histology, and diagnosed within maximally 36 months after blood donation. Data on tumor histology were available for all 197 cases, whereas data on tumor grade and stage were available for 133 (68%) and 180 (91%) of the cases, respectively. A

further description of data collection in EPIC on basic risk factors, prospective cancer incidence and tumor characterization is in the **Supplemental Methods**.

For each of the 197 case subjects up to four control participants (N=725) were randomly selected among appropriate risk sets consisting of all female cohort members with a blood sample, alive and free of cancer at the time of diagnosis of the index case. An incidence density sampling protocol was used, such that, in principle, control participants could include women who became a case later in time and each control participant could be sampled more than once; however, none of the control participants have subsequently been identified as ovarian cancer cases. Case and control participants were matched on study recruitment center, age at blood donation (±6 months), time of the day of blood collection (±1 h), fasting status (<3 h, 3–6 h, >6 h), follow-up time, and menopausal status at blood collection, use of oral contraceptives or post-menopausal hormone replacements at the time of blood draw, and phase of menstrual cycle for premenopausal women.

Laboratory assays

Serum samples were analyzed in batches, sorted by study center and with samples from matched case-control sets together in the same batch. Measurements of CA125 were performed in the Genital Tract Biology Lab at Brigham Women's Hospital, Boston, using a highly sensitive electrochemo-luminiscence (ECL) detection platform (Meso Scale Discovery, MSD), following methods described in detail previously **(5)**. Measurements of AAbs were performed at Virginia G. Piper Center for Personal Diagnostics, Biodesign Institute, Arizona State University, using Rapid Antigenic Protein In situ Display (RAPID) ELISA as previously described **(18)**. The proteins were expressed as c-terminal GST fusion proteins using 1-Step Human Coupled *in vitro* Expression system (Thermo Scientific) and added to 96 well plates. Patient serum was diluted 1:100 in blocking buffer, and bound IgG antibody was detected using HRP conjugated goat anti-human IgG (Jackson ImmunoResearch Laboratories) and

Supersignal ELISA Femto Chemiluminescent Substrate (Thermo Scientific). Relative light unit (RLU) ratios were calculated using the RLU of a specific antigen divided by the RLU of the control GST-protein. All assays were performed in duplicate and the average level was used. All samples were blinded to the investigators.. Measurements of CA125 and AAbs were completed for a total of 194 incident cases of invasive ovarian cancer and 705 matched, cancer-free control participants. Missing values were due to insufficient sample volume for the AAb assays (6 samples, including 2 cases), and to missing data for previous measurements of CA125 (1 further case and 16 further controls).

Statistical analyses

Detection sensitivities were calculated at quantitative marker cut-off points corresponding to 95% (SE95) and 98% (SE98) specificity, respectively, determined on raw and adjusted biomarker values among all control participants (N=705).

The biomarker values were separately adjusted through linear regression models, fitted to the full control population, using country, age, menopausal status and use of either oral contraceptives (OC) or menopausal hormone replacement (HRT) at blood draw as predictors. Those linear adjustment models were applied to all sample subjects and the markers' residuals added to the markers' overall mean values, before further analyses by unconditional logistic regression. As findings from adjusted and un-adjusted marker analyses were practically identical, however, only the basic results from unadjusted analyses are presented.

Logistic regression modelling was used further for analyses of receiver operating characteristic (ROC) curves and C-statistics, and to examine the discrimination capacity of multiple markers in combination. For multi-marker discrimination models, the statistical fit of nested models was compared using likelihood-ratio tests. In ROC analyses, the area under curve (AUC; also

referred to as concordance [C-]statistic) was calculated as an overall measure for the markers' capacity to discriminate future cancer cases from participants.

All analyses were performed by strata of lag-time (≤6, >6-12, >12-24, and >24-36 months), and were conducted in SAS, version 9.4 (SAS Institute, Cary, NC, USA).

Informed consent and data protection

All EPIC study participants had given their consent for future analyses of their blood samples for research purposes and the present study was approved by the IARC Ethics Committee and the Institutional Review Boards of Brigham and Women's Hospital and of the University of Heidelberg.

Results

For the 194 ovarian cancer cases and 705 matched control participants with complete biomarker measurements, baseline and tumor characteristics are presented in **Table 1**. Overall, the median age at cancer diagnosis was 59 years (range: 31–79 years). Of the 194 cancer cases, 187 (96%) had the ovary classified as primary tumor site, whereas in 4 (2%) the primary site was the fallopian tube and in 3 patients (3%) it was the peritoneum. More than half of the tumors (56%; n=108) were of serous histology. Of the 178 cases with stage data available, 32 were diagnosed with localized disease, whereas the remainder (N=146) were coded as having advanced (regionally spread and/or metastatic) disease.

Adjusting for age and study center, partial (Spearman) correlation analyses revealed no significant associations between CA125 and any of the AAb markers among the controls; however, among the cases there were weak but significant associations of CA125 with AAbs against CTAG1A (r=0.17) and p53 (r=0.18). Furthermore, there were significant and moderately

strong correlations across the four AAbs, ranging from 0.38 to 0.62, both among the cases and the controls (**Supplemental Figure S1**). Cross-sectional analyses revealed no strong correlations (all estimated values <0.13) for any of the AAbs with age or menopausal status at blood draw, parity, age at last child birth, estimated lifetime number of ovulatory cycles, BMI, smoking, or serum levels of C-reactive protein (CRP) as a biomarker of inflammation status (results not shown).

Box and whisker plots in (**Figure 1**) show that for CA125 levels started diverging between future cases and control participants about 24 months prior to clinical diagnosis, and this difference grew larger as the lag-time diminished to 6 months or less, with a corresponding increase in the proportion of cases with marker levels above the 95% or 98% specificity cut-points. For each of the AAb markers the plots show similar trends of increasing proportion of ovarian cancer cases with elevated AAb titers as lag-times shortened, although absolute numbers of cases reaching threshold titers for 95% or 98% detection specificity were modest. Interestingly, the Box and whisker plots also showed elevated right-tail AAb titers in non-negligible proportions of cancer-free control participants.

Using a quantitative marker cut-point corresponding to 98% specificity, CA125 showed sensitivity estimates (SE98) of 0.77, 0.34 and 0.20, respectively, for lag-times ≤6, >6-12, and >12-24 months, whereas for lag-times >24-36 months the sensitivity (SE98) was close to zero (0.03) (**Table 2**). For the AAb markers, estimates of SE98 ranged from 0.19 (CTAG1A, CTAG2, NUDT11) to 0.23 (P53) within the first 6 months after blood donation, from 0.03 (CTAG1A, NUDT11) to 0.11 (P53) for serum samples taken >6-12 months prior to diagnosis, and from 0.01 (NUDT11) to 0.11 (CTAG1A) for serum samples drawn >12-24 months prior to diagnosis. Using more lenient 95% specificity cut-points, the estimated sensitivities (SE95) were slightly higher.

When analyses were restricted to high-grade- serous tumors, estimates for SE98 or SE95 were slightly higher for the AAb against P53 (e.g. SE98 = 0.33 and 0.17 for ≤6 and >6-12 months, respectively), but not for the other AAbs, whereas for all AAbs (including those to P53) early detection sensitivities (SE98 or SE95) remained practically zero for longer time lags (**Table 2**).

Among the control participants, a total of 61 women developed cancer over an extended follow-up of up to 20 years after blood donation, including one case of breast cancer within ≤36 months and one case of melanoma within ≤60 months. Excluding these control participants did not materially change estimates for 95% and 98% specificity cut-points, nor did it change estimates of SE98 or SE95 for early ovarian cancer detection.

Considering blood measurements ≤24 months before clinical diagnosis (the time frame within which marker discrimination could be most clearly observed), and using 98% specificity cut-points for each of the five markers, 47 out of 137 future cases of ovarian cancer (34%) showed positive test findings for CA125. Of the 82 CA125-negative cases, 8 (9.8%) would have been additionally detected through any one of the four AAbs. All 8 cases all had blood samples predating clinical diagnosis by >6-24 months – a lead-time window in which the diagnostic sensitivity of CA125 was lower, and in which a larger proportion of tumors may have been still in earlier stages (**Table 3**). However, a combined diagnostic algorithm based on positive tests for either CA125 or any of the four AAbs would have also increased the false-positive detection rate [FPR] among controls to 8.4%. Setting the quantitative specificity cut-point for CA125 to the same level yielded an equivalent increase in detection sensitivity for CA125 alone. Focusing on CTAG1A-AAb only, the one AAb marker that detected the largest proportion (6 of the 8) CA125-negative cases, the overall FPR for joint detection by either CA125 or AAb was lower (4.3%); still, the reduced panel of CA125 and CTAG1A-AAb did not outperform CA125 with a cut-point set at an equivalent FPR (e.g., sensitivity at FPR of 4.3% for lead time >12-24 months, CA125 or CTAG1A positive: 20%; CA125 alone: 19%) (**Table 3**). Similar results were observed for

other marker combinations (**Table 3**) or with marker cut-points corresponding to either higher (99%) or lower (95%) levels of specificity.

Still focusing on data for the first 24 months of prospective follow-up, when modelling all markers on a continuous (log2-transformed) scale by logistic regression the overall model fit improved significantly ($p=0.003$) when the four AAbs were added to a model including CA125, but with only very modest increases in AUC (from 0.78 for CA125 alone, to 0.80 for the full model) (**Table 4A**). A backward elimination strategy, eliminating markers not contributing significantly to the model at a significance level of $p \leq 0.10$, resulted in a model containing only CA125, CTAG1A and NUDT11 that retained most of the improvement in model fit and in the AUC. Entering the AAbs as variables dichotomized around their 98% specificity cut-points led to a similar model selection of CA125 plus CTAG1A only, with similarly modest increases in AUC. In none of the above models, however, was there any improvement in detection sensitivity at overall 95% or 98% specificity for the corresponding relative risk (logistic regression) scores.

Discussion

In this prospective study, a panel of four selected tumor-associated autoantibodies showed selectivity, but limited sensitivity, for early detection of ovarian cancer, prior to diagnosis under usual care. In serum samples predating symptomatic diagnosis by less than 6 months, each individual AAb marker showed a diagnostic sensitivity (SE98) of around 0.20 at 0.98 specificity, similar to levels of diagnostic sensitivity observed in cross-sectional comparisons between clinically diagnosed ovarian cancer patients and cancer-free controls (9). However, the ability of these AAb markers to distinguish cases from controls declined rapidly with time between blood draw and diagnosis, and SE98 estimates were close to zero in serum samples collected at greater than 1-year lead times. These observations suggest that high AAb titers to these selected cancer-associated antigens may represent increasing tumor burden, possibly related to increasing inflammation and immune cell infiltration, and that serial measurements may be needed to improve diagnostic performance. Combined logistic regression scores of the AAbs and CA125 showed no meaningful improvement in diagnostic discrimination (AUCs, SE98) compared to CA125 alone, despite a statistically significant improvement in overall model fit.

The AAbs included in the present study were selected on the basis of their diagnostic performance in previous studies by both our own **(10-12, 14, 18)**, and other research groups **(13)** (19). Elevated serum P53 AAbs are observed in relation to many other cancer types, including lung, breast and gastro-intestinal tumors **(6-8)**, and elevated AAb titers to P53 have also been observed in more than ten studies comparing ovarian cancer patients to cancer-free control subjects (reviewed in **(9)**). Generally, the studies on ovarian cancer reported higher prevalence of elevated P53 AAbs among patients with high-grade serous tumors, as compared to other tumor subtypes, as was also observed in the current study. The higher sensitivity and specificity of P53 AAb for high-grade serous tumors is likely related to the uniform occurrence of P53 mutations, with dysregulated P53 protein levels, in high-grade serous tumors. Like the P53

AAbs, elevated titers of AAbs to the cancer-testis antigens CTAG1A (NY-ESO-1) and CTAG2 (ESO2) have also been observed in relation to a wide variety of cancer types **(6-8) (14, 15)**, including ovarian cancer **(20) (9) (13)**, and are likely related to the generally less differentiated nature of cancer cells, with aberrant expression of proteins that normally are expressed only in embryonic tissue types. AAbs to NUDT11 were first discovered as ovarian autoantigens through our own immuno-proteomic screening of ovarian cancer patients and controls **(18).**

In clinical studies comparing cancer patients (ovary and other organ sites) with cancer-free controls, strongly skewed distributions of AAbs with elevated right-tail values for cancer patients have suggested high cancer-diagnostic specificity of high antibody titers. However, for our selected panel of AAbs we also observed a non-negligible prevalence of elevated “right-tail” titers among control participants plus, surprisingly, moderately strong correlations across all four AAbs among both cases and controls. Exclusion of controls with a cancer diagnosis during extended follow-up did not alter this pattern. Thus, our data suggest there may be substantial structural variation in autoantibody titers among individuals, independent of tumor development. While the biological mechanisms that may underlie this variation remain unclear (i.e., we observed no strong correlations between the AAbs and standard cancer risk factors), our combined observations do suggest that AAbs against P53, CTAG (“cancer-testis antigens”) or other antigens considered to be tumor associated may have lower cancer specificity than is generally assumed.

One other prospective evaluation of AAbs as early detection markers for ovarian cancer was reported recently for P53-AAbs **(17)**. This study by Yang et al. was based on analyses within the multimodal screening arm of UKCTOCS – a population-based, randomized trial of ovarian cancer screening among post-menopausal women in the United Kingdom -- and included 220 ovarian cancer cases with 1,053 serial serum samples collected up to 5 years prior to ovarian cancer diagnosis, and 619 age-matched ovarian cancer-free controls with sera collected

annually (n=3,069 samples). The majority of the ovarian cancer cases (74.5%) were screen-detected using CA125 and the ROCA algorithm followed by TVUS; the remainder (25.5%) were screen negative cases. Applying a P53-AAb cut-point corresponding to 2.7% specificity, Yang et al. reported a positive P53 antibody signal in 20.7% of the screen-positive cases and 16.1% of the screen-negative cases. Further, among screen-positive cases, P53 was elevated an average of 9.2 months prior to detection by ROCA, or 8.1 months prior to elevated CA125 (>35 U/mL) alone. Likewise, a P53-AAb signal was also observed among 9 of the 56 screen-negative cases (16.1%). However, the authors did not report the overall false-positive rate associated with a diagnostic algorithm based on the combinations of P53-AAb with either ROCA or single-time elevation of CA125, nor did they report whether a similar improvement in OC detection could have been achieved on the basis of CA125 measurements only at an equivalent relaxation of specificity (i.e., using lower-specificity marker cut-points for either ROCA or single-time CA125). In our data, generated by a different ELISA assay method for P53-AAb, while we also observed positive AAb signals (notably against CTAG1A) in a proportion of future ovarian cancer testing negative by CA125, further analyses showed that diagnostic algorithms based on combinations of CA125 with AAbs did not actually outperform CA125 alone at equivalent false-positive detection rates.

As a further analysis within the UKCTOCS, Yang et al performed multivariate logistic regression and ROC curve analyses to examine combined detection capacity of CA125 (single-time measurement at [98.1% specificity] cut-point of 35 U/mL) and P53-AAb. As in our study, they observed a statistically significant improvement in model fit and a modest increase in overall AUC for the combined, two-marker model as compared to a model based on CA125 only. However, as in our data, there was no improvement in at 98% specificity. Furthermore, ovarian cancer diagnoses in the UKCTOCS multi-modal screening arm were largely driven by ROCA analyses of longitudinal changes in CA125. This introduces a methodologic complication for

analyses of a single measure of CA125 alone, given the ROCA algorithm has higher sensitivity than a one-time measurement of CA125, and may have effectively handicapped the performance of a single measure of CA125, with possible overestimation of the complementary detection potential for P53-AAb.

In conclusion, our selected AAbs were confirmed as potential biomarkers for the detection of ovarian cancer, but they did not appear to provide a meaningful improvement over CA125 alone in this sample set. Furthermore, diagnostic discrimination of the AAbs appears to wane with longer lead times between blood collection and diagnosis, suggesting that AAbs against these cancer-related antigens may have limited utility for very early lesions. An unexpected finding was the non-negligible prevalence of high AAb titers among the cancer-free controls, which appears to put a possible limit to the specificity that these AAbs may have as cancer detection markers.

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